SOX2 Gene Amplification and Overexpression is Linked to HPV-positive Vulvar Carcinomas.

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Abstract: SOX2 (SRY-related HMG-box 2) belongs to the SOX gene family of high-mobility transcription factors indispensably involved in gene regulation in pluripotent stem cells and neural differentiation. SOX2 copy number increases have been frequently reported in various types of squamous cell cancer. To better understand the effect of SOX2 aberrations on vulvar cancer phenotype and patient prognosis, we analyzed SOX2 copy number changes using fluorescence in situ hybridization and SOX2 expression by immunohistochemistry in 55 squamous cell carcinomas of the vulva. SOX2 amplification was found in 20.8% of tumors; 27.3% of vulvar carcinomas showed SOX2 protein overexpression. SOX2 amplification was correlated with SOX2 overexpression in our data set ($P<0.01$). Amplification of the SOX2 locus was associated with high tumor grade ($P<0.05$) and human papillomavirus (HPV) positivity ($P<0.01$). SOX2-amplified tumors showed more frequently a basaloid phenotype than nonamplified carcinomas. SOX2 protein overexpression was also correlated with basaloid phenotype and positive HPV status of vulvar carcinomas ($P<0.05$, each). SOX2 amplification and expression were not associated with patient overall survival. In conclusion, SOX2 copy number increases are detectable in a substantial proportion of high-grade HPV-positive vulvar carcinomas with basaloid differentiation. Our study provides further evidence for different molecular alterations in HPV-positive and HPV-negative vulvar carcinomas.

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Original Article

SOX2 Gene Amplification and Overexpression is Linked to HPV-positive Vulvar Carcinomas

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Summary: *SOX2 (SRY-related HMG-box 2)* belongs to the *SOX* gene family of high-mobility transcription factors indispensably involved in gene regulation in pluripotent stem cells and neural differentiation. *SOX2* copy number increases have been frequently reported in various types of squamous cell cancer. To better understand the effect of *SOX2* aberrations on vulvar cancer phenotype and patient prognosis, we analyzed *SOX2* copy number changes using fluorescence *in situ* hybridization and SOX2 expression by immunohistochemistry in 55 squamous cell carcinomas of the vulva. *SOX2* amplification was found in 20.8% of tumors; 27.3% of vulvar carcinomas showed SOX2 protein overexpression. *SOX2* amplification was correlated with SOX2 overexpression in our data set ($P<0.01$). Amplification of the *SOX2* locus was associated with high tumor grade ($P<0.05$) and human papillomavirus (HPV) positivity ($P<0.01$). *SOX2*-amplified tumors showed more frequently a basaloid phenotype than nonamplified carcinomas. SOX2 protein overexpression was also correlated with basaloid phenotype and positive HPV status of vulvar carcinomas ($P<0.05$, each). *SOX2* amplification and expression were not associated with patient overall survival. In conclusion, *SOX2* copy number increases are detectable in a substantial proportion of high-grade HPV-positive vulvar carcinomas with basaloid differentiation. Our study provides further evidence for different molecular alterations in HPV-positive and HPV-negative vulvar carcinomas. **Key Words:** SOX2—Vulvar cancer—Amplification—prognosis.

Gene amplification is a key mechanism for protein overexpression and oncogene activation in tumor cells (1). Amplified oncogenes are markers of general genomic instability as the base for the development of further genomic aberrations. Recurrent chromosomal gains in vulvar cancer have been found at 1q, 3q, 4p, 5p, 8p, 8q, and 12q (2–6). Possible candidate targets at 3q are *human telomerase RNA* and the *PIK3CA* gene. *MYC* oncogene is the potential target of the 8q amplification in vulvar cancer. Growdon et al. (7) observed *EGFR* amplifications in 12% of vulvar carcinomas with adverse prognosis.

The *SOX2* gene is located on chromosome 3q26.33 and codes for a 317-amino acid protein that belongs to the *SOX2* gene family of transcription factors (8). Together with other proteins SOX2 is crucial in the maintenance of pluripotency in embryonic stem cells (9). In cooperation with Oct4, c-Myc, and Klf4, SOX2 is capable of reprograming differentiated cells into an induced pluripotent stem cell-like phenotype (10). Amplification and overexpression of *SOX2* has been reported in a wide variety of cancers, including squamous cell carcinomas (SCC) of various origins (11). Besides genomic alterations, human papillomavirus (HPV) infection plays an

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important role in vulvar carcinoma initiation (12). Approximately one third to two thirds of all vulvar tumors are related to HPV (predominantly HPV type 16) (13). However, a substantial proportion of vulvar cancers are not HPV related, especially in elderly women (14). HPV-negative tumors are frequently *TP53* mutated, as shown in former studies (15). Although still controversial, it is generally assumed that the prognosis in HPV-related tumors is better than that in non-papillomavirus-associated carcinomas.

To improve our understanding of *SOX2* copy number increases and protein expression in vulvar cancer, we analyzed 55 vulvar tumors with clinical follow-up data by immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH). The results suggest a possible role of *SOX2* copy number changes in the progression and development of HPV-related vulvar carcinomas.

METHODS

A total of 55 formalin-fixed (buffered neutral aqueous 4% solution), paraffin-embedded squamous cell vulvar carcinomas were available from the Department of Pathology and Molecular Pathology, University Hospital Zurich, Switzerland, between 2008 and 2014. The median patient age was 68.9 years (range, 37–89 y). Data were retrieved from patients' records, the tumor registry, and death certificates. Clinicopathologic factors were evaluated by reviewing medical charts and pathologic reports. Clinical outcome was followed up from the date of primary surgery to the date of death or last documented visit in our archive system. Permission for performing the study was obtained from the local ethics committee.

The histology of all tumors was reevaluated by 1 experienced gynecopathologist (M.C.) and classified according to WHO as keratinizing SCC, nonkeratinizing SCC, and basaloid SCC (16,17). The criteria for classification as basaloid SCC were composition of immature cells with scanty cytoplasm resembling basal cells of the epidermis and no or only little keratinization. The tumors were graded according to recommendations of the Gynecologic Oncology Group into 3 grades: G1, no undifferentiated cells; G2, $<50\%$ undifferentiated cells; and G3, $\geq 50\%$ undifferentiated tumor cells (18).

Tissue microarray construction was as described previously (19). Briefly, tissue cylinders with a diameter of 0.6 mm and a height of 3 to 4 mm were punched from representative tumor areas of a "donor" tissue block using a custom-built instrument. They were then brought into 1 recipient paraffin block containing 2 representative tissue spots of 55 individual samples.

SOX2 FISH Analysis

The probe for analysis of *SOX2* was provided by ZytoVision GmbH (Germany). The probe combination was *SOX2* (ZyGreen)/centromere 3 (ZyOrange). Slides dedicated to dual-color FISH analysis were prepared according to recommendations of the manufacturer. Evaluation of *SOX2* amplification status was performed as follows: for each tissue spot, the predominant gene and centromere copy numbers in the tumor cell nuclei were estimated. A tumor was considered amplified if the ratio of gene/centromere was ≥ 2.0 or by the presence of gene clusters. Ratios >1.0 and <2.0 were considered as gains and a ratio ≤ 1.0 was considered normal (20).

SOX2 IHC

Commercially available antibodies raised against SOX2 (clone EPR3131, 1:100; Epitomics Inc.) were used on a Ventana Benchmark automated staining system (Ventana Medical Systems Inc., Tucson, AZ). For SOX2, only nuclear staining was regarded as specific. IHC was evaluated in 2 cores per tumor. SOX2 staining intensity and the fraction of stained tumor cells were recorded for each tissue spot. Staining intensity was estimated on a 4-step scale (0, no staining; 1+, faint intensity; 2+, moderate intensity; 3+, strongest intensity). The fraction of stained cells was scored according to the following criteria: score 0, no stained cells; score 1, $\leq 25\%$ stained cells; score 2, $\leq 50\%$ stained cells; score 3, $\leq 75\%$ stained cells; and score 4, $>75\%$ stained cells. A final IHC result was obtained from these scores: negative, no staining at all; weak, intensity 1+, or intensity 2+ in $\leq 50\%$ of cells, or intensity 3+ in $\leq 25\%$ of cells; moderate, intensity 2+ in 50% to 75% of cells, or intensity 3+ in 25% to 50% of cells; strong, intensity 2+ in $>75\%$ of cells, or intensity 3+ in $>50\%$ of cells. Both negative and weak as well as moderate and strong SOX2 expressions were combined with low-level and high-level expression patterns for further analyses.

HPV Detection

Analysis of the tumor HPV status was carried out with RNA in situ hybridization, utilizing specific

probes against pan HPV and HPV 16 [ViewRNAeZ-L ISH detection kit (PN 19500) from Affymetrix Inc., Santa Clara, CA]. HPV 16 (PN DVA1-17255) targeted E6/E7 of HPV 16 and pan HPV (PN DVA1-17029) targeted E6/E7 of 14 high-risk HPV strains (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). All probe sets were diluted 1:40, except the pan HPV 1 (1:200); the probes were hybridized for 3 hours using the "View RNA red detection" protocol on the Leica Bond RX (21). Appropriate positive and negative controls were applied by manufacturer recommendations.

Statistics

The Pearson χ^2 test and Student *t* test were used to study the relationship between SOX2 and clinicopathologic parameters. The survival effect of SOX2 was assessed by the Kaplan-Meier curves and log-rank tests. Analysis was performed using R statistical software package for Windows (version 2.7.2, R Foundation for statistical computing).

RESULTS

SOX2 FISH and IHC

A total of 48 cancer samples were assessable by FISH on the tissue microarray (Table 1). Overall, *SOX2* copy number increases were found in 10 (20.8%) vulvar cancers. Figure 1 displays a representative example with *SOX2* amplification. All tumors with copy number increases showed low-level amplification with a signal ratio of 2 to 10 per nucleus. Immunohistochemical examination of SOX2 expression was possible in 55 vulvar carcinomas (Table 1). The expression pattern was always nuclear. Figures 2A–C illustrate representative examples with low-level and high-level SOX2 expression according to our predefined criteria. An overall 27.3% of vulvar carcinomas showed a strong SOX2 expression. SOX2 expression was statistically significantly related to *SOX2* gene copy number in the present cohort of vulvar cancers. Seven out of 10 vulvar cancers with *SOX2* amplification exhibited a strong nuclear SOX2 expression signal ($P<0.01$, Table 2). However, high-level protein expression was also detectable in 6 of 32 vulvar carcinomas without *SOX2* amplification.

Association With Clinicopathologic Features

Table 1 summarizes the association of *SOX2* copy number increases and SOX2 protein expression with clinicopathologic parameters of vulvar carcinomas. *SOX2* copy number increases were related to tumor grade and HPV status of vulvar carcinomas. Amplification of *SOX2* was more frequent in poorly differentiated ($P<0.05$; G1 + G2 vs. G3) and HPV-positive ($P<0.05$) tumors. There was a tendency for *SOX2*-amplified cancers to show more frequently a basaloid phenotype, but this result was not significant (20% vs. 7.9%). However, we observed a statistically significant connection between SOX2 protein overexpression and basaloid phenotype of vulvar carcinomas ($P<0.05$). Furthermore, SOX2 expression was linked to positive HPV status of vulvar cancer ($P<0.05$). There was no statistical correlation between higher tumor grade and HPV positivity (50% vs. 24.3%). The last result did not reach statistical significance.

There was also no association of *SOX2* copy number alterations or SOX2 expression with patient age, pN category, or tumor stage.

We examined the relationship between SOX2 expression and amplification in vulvar carcinomas and overall survival of vulvar cancer patients. There was no clear influence of these parameters on overall survival in our analysis (Fig. 3).

DISCUSSION

In our study, we detected frequent *SOX2* amplification and SOX2 protein overexpression in vulvar carcinomas. *SOX2* copy number increases correlated with SOX2 overexpression, suggesting that *SOX2* gene amplification is the main mechanism for SOX2 overexpression in vulvar cancer. Interestingly, 6 tumors with SOX2 overexpression were not amplified in our analysis. In these carcinomas other mechanisms than amplification are presumably responsible for SOX2 upregulation. Activity of SOX2 is controlled by various translational and posttranslational modifications. At the translational level, inhibitory microRNAs play a pivotal role and could be a mechanism for elevated SOX2 levels in the absence of amplification (22).

At present, there are no data available in the literature on *SOX2* amplification in vulvar cancer, but our observations are comparable to previous reports on SCC of the esophagus, lung, head and neck, and other localizations (20,23–25). These studies describe similar ranges for *SOX2* amplification of between 11% and 32% of examined tumors as found in our study for vulvar cancer. Data on SOX2 protein overexpression are also limited in vulvar

TABLE 1. *Relationships between SOX2 copy number as well as SOX2 expression and clinicopathologic features in vulvar carcinomas*

		SOX2 FISH		SOX2 IHC		
	All (n)	Disomy (n)	Amplification (n)	Low Level (n)	High Level (n)	*P*
Samples	55	38	10	40	15	
Age (y)						
Median	69	70	63.5	68	70	NS
Histologic tumor type						
Keratinizing	26	20	4	21	5	NS/$P<0.05$
Nonkeratinizing	23	15	4	18	5	
Basaloid	6	3	2	1	5	
Tumor stage						
pT1a	4	3	1	3	1	NS
pT1b	31	20	6	24	7	
pT2	10	9		7	3	
pT3	4	4		2	2	
pT4	3	1	2	1	2	
Nodal stage						
pN0	17	12	3	11	6	NS
pN1a,b	5	4		5		
pN2a-c	10	6	3	8	2	
Grading						
G1	6	6		6	0	$P<0.05$/NS
G2	29	23	2	22	7	
G3	16	7	6	9	7	
HPV						
Negative	25	19	2	21	4	$P<0.05$
Positive	21	12	8	11	10	

FISH indicates fluorescence *in situ* hybridization; HPV, human papillomavirus; IHC, immunohistochemistry; NS, not significant.

carcinomas. Brustmann and Brunner (26) examined SOX2 expression in a cohort of 33 vulvar carcinomas and precursor lesions. In their study, SOX2 was expressed in 72.7% of invasive carcinomas compared with only 27.3% in our present analysis. This difference may be due to the smaller sample size of the other cohort and our tissue microarray approach, which could lead to identification of a lower expression frequency. Interestingly, Brustmann and Brunner also observed SOX2 overexpression especially in poorly differentiated carcinomas. Our immunohistochemical SOX2 expression results are comparable to those of oral SCC (24,27).

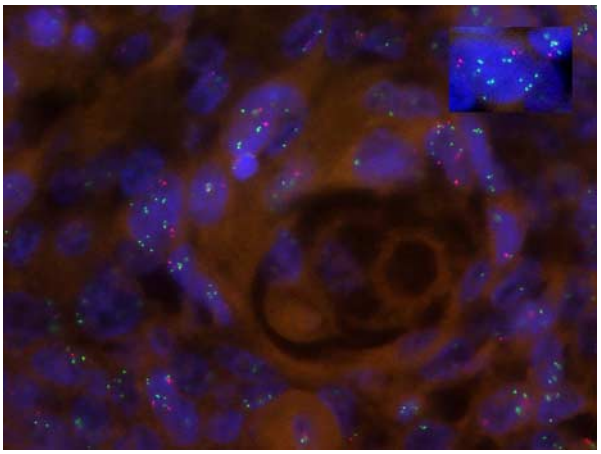

FIG. 1. Keratinizing squamous cell carcinoma of the vulva showing low-level amplification of the *SOX2* gene: red signals indicate copy number of chromosome 3 and green signals indicate *SOX2* copy number. (Magnification ×400 with zoomed-up inset).

The proportion of 45.7% HPV-associated carcinomas in our cohort was similar to that in previous studies (28–30). There was a striking link between *SOX2* amplification/overexpression and HPV positivity of vulvar carcinomas in our analysis. Furthermore, immunohistochemically detectable SOX2 upregulation was connected to basaloid differentiation of vulvar carcinomas. The known relationship between HPV, basaloid phenotype of carcinomas, and poor differentiation was clearly reproducible in our data set (31). Thus, the connection of *SOX2* amplification and overexpression to basaloid and poorly differentiated carcinomas also argues toward a possible relationship between HPV status and *SOX2* aberrations in vulva tumors. Interestingly, similar SOX2 expression levels were found in morphologically and immunohistochemically characterized vulvar intraepithelial neoplasias of classic (HPV associated) and differentiated types (HPV independent), respectively (26). However, we detected *SOX2* overexpression and amplification only in a minor subset of HPV-negative invasive vulvar carcinomas.

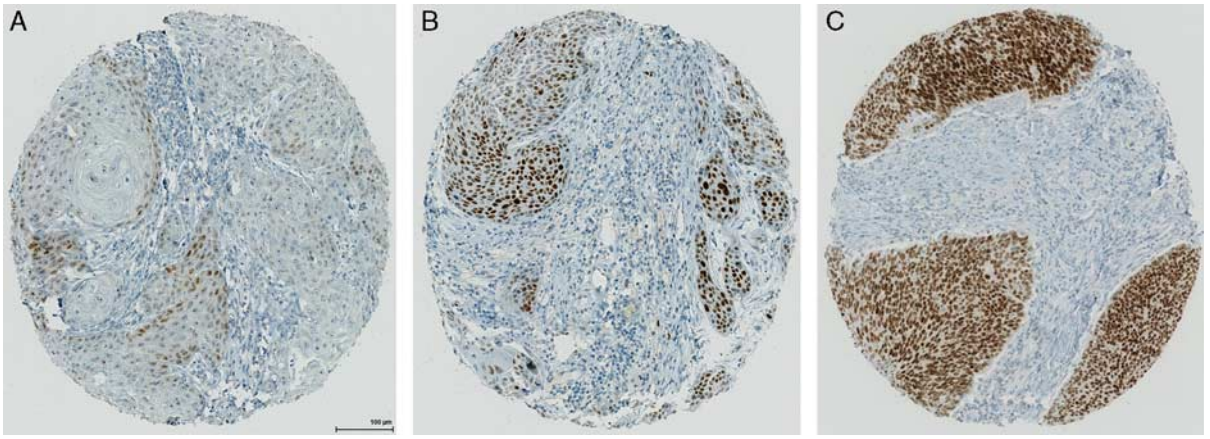

FIG. 2. Examples of SOX2 immunohistochemistry results in vulvar carcinomas: weak (A), moderate (B), and strong (C) expression.

Molecular characterization of HPV association of invasive carcinomas in the present study may explain the analytical differences (32).

The molecular events in HPV-driven vulvar tumors and HPV-negative carcinomas are quite different. Amplifications of canonical cancer genes such as *EGFR* and *CCND1* seem to mostly arise in HPV-unrelated carcinomas (33). In this type of vulvar carcinoma, *TP53* mutations play a crucial role in cancer progression. HPV-related tumorigenesis is driven by viral oncogenes E6 and E7 that disrupt the retinoblastoma pathway in tumor cells. However, there are some hints that the insertion of HPV in the host genome is not random. Studies in head and neck carcinomas showed possible relations between insertion loci and amplification of associated genes, whereas only low levels of E6 or E7 are observed (34). These observations could explain the preferential amplification of specific genes, including *SOX2*, in HPV-related cancers. In addition, the linkage between *SOX2* amplification and G3 status of vulvar cancer may be the sequel of genomic instability in these cancers.

The 2016 WHO classification newly separated penile carcinomas in HPV-positive and HPV-negative SCC. Subtypes of HPV-positive penile carcinomas were basaloid SCC, warty SCC, and warty-basaloid SCC. Our study confirmed that the basaloid phenotype is linked to HPV infection in vulvar SCC also. Data on molecular alterations in penile carcinomas are limited (35). Recently we could demonstrate that amplification and overexpression of *CCND1* at 11q is related to HPV-negative vulvar carcinomas (36). Therefore, it is tempting to speculate that *SOX2* alterations are more relevant for HPV-positive SCC, whereas *CCND1* is involved in the progression of HPV-negative SCC in vulvar and in penile cancer (37). A future WHO classification of vulvar SCC could be therefore adapted to the penile WHO classification.

In summary, the data of this study show that *SOX2* is variably amplified in vulvar cancers and is related to HPV-driven carcinogenesis in vulvar carcinomas.

TABLE 2. *Correlation of SOX2 copy number alterations and the respective protein expression in vulvar carcinomas*

	SOX2 FISH		
	Disomy (n)	Amplification (n)	*P*
SOX2 IHC			
Low level	32	3	<0.01
High level	6	7	

FISH indicates fluorescence *in situ* hybridization; IHC, immunohistochemistry.

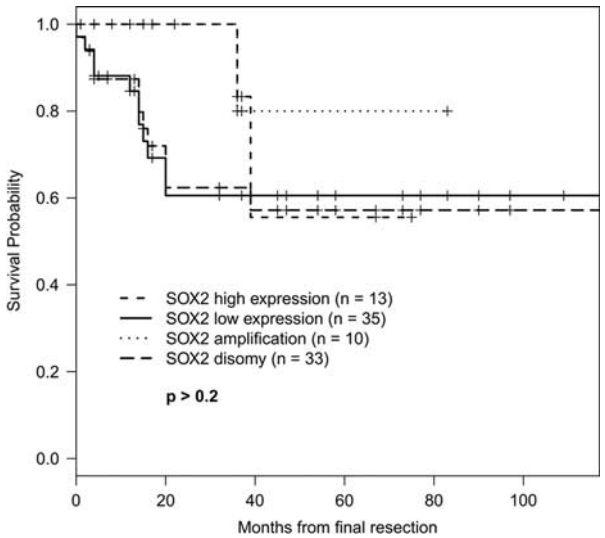

FIG. 3. Prognostic significance of *SOX2* amplification and SOX2 expression level in vulvar carcinomas.

The specific role of SOX2 in combination with other lineage-specific genes in SCCs makes it suitable as a therapeutic target in a broad range of cancers, including vulvar carcinoma (38).

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